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PROVISIONAL APPLICATION COVER SHEET

Seattle, Washington 98101

December 19, 2003



TO THE COMMISSIONER FOR PATENTS:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

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- X a. complete
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provisional patent application of inventor(s):

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Title of Invention: A genetically attenuated malaria parasite

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- X 1. A provisional application for patent consisting of 18 pages of specification and drawings is enclosed.
- X 2. Small entity status is asserted in this matter.
3. An Assignment of the invention to _____ is enclosed, together with a Recordation Cover Sheet in accordance with 37 C.F.R. § 3.31 and our check including the amount of \$40 to cover the recordation fee. Please record this Assignment in accordance with 37 C.F.R. § 3.11.

A genetically attenuated malaria parasite as a non-infectious whole organism vaccine that confers sterile protection

Malaria is a parasitic disease infecting 300-500 million and killing 2 million people each year. It is also the number one infectious threat facing the US soldier, and is the leading cause of all casualties during tropical deployments. In addition, malaria poses a great danger to tourists traveling in tropical countries. The *Plasmodium* parasites causing malaria become increasingly resistant to the small arsenal of available drugs, and there is no vaccine on the market. Thus, the situation is likely to worsen in the years to come. *Plasmodium* parasites are transmitted to the vertebrate host by mosquito bite, which inoculates the infectious sporozoite stage. Plasmodia are obligatory intracellular parasites and their progression through the life cycle involves invasion and intracellular replication in different types of host cells. When sporozoites are injected into the mammalian host together with the saliva of a biting mosquito they quickly enter the blood circulation and home in to the liver where they exit the blood vessel and invade hepatocytes. Here the parasite becomes a liver stage. The liver stages are the obligatory bridgehead between the sporozoite stage in the mosquito vector and the initiation of red blood cell infection by merozoites in the mammalian host. The spherical liver stages grow, undergo multiple rounds of nuclear division and finally differentiate into thousands of first generation merozoites. Thus, a few days following sporozoite invasion thousands of merozoites are released from the liver. They rapidly invade red blood cells and initiate the erythrocytic cycle, which causes the typical symptoms of malaria infection. Preventing liver stage development prevents malaria infection.

An experimental vaccine that consists of gamma radiation-attenuated sporozoites elicits protective immunity in humans and animal models (Hoffman and Doolan, 2000; Hoffman *et al.*, 2002; Nussenzweig and Nussenzweig, 1989). The attenuated parasites infect hepatocytes and initiate liver stage development however, they cannot replicate (Nussler *et al.*, 1989; Sigler *et al.*, 1984). The irradiation dose is very critical for the success of this experimental vaccine. A low dose will not prevent DNA replication further liver stage development, and onset of malaria disease. A high dose of irradiation will kill the parasites and prevent early development, thereby preventing priming of the immune response. Because of these difficulties and because no other means have been available to properly attenuate the parasite, it has never been considered feasible to develop irradiated sporozoites into a vaccine.

We have created a *Plasmodium berghei* parasite (a rodent malaria parasite used as a model to study malaria) that is genetically attenuated at the sporozoite stage. This was done by targeted disruption of a gene (knockout, ko) named *S3/UIS4* (the mutant *P. berghei* parasite clone is herein referred to as: S3/UIS4 ko). The gene disruption (Ménard and Janse, 1997) was done using insertion of a selectable marker into the S3/UIS4 locus (Figure 1).

The S3/UIS4 (accession number: EAA21410) gene was previously identified by using differential gene expression screens with the related rodent malaria parasite species *P. yoelii* (see appended paper in press, which is incorporated by reference herein). The

S3/UIS4 gene is expressed in salivary gland sporozoites but is not expressed in the parasites blood stages (see appended paper in press).

Here, we show that S3/UIS4ko parasites show normal development of the mosquito stages of the parasite and develop normal numbers of sporozoites in the mosquito salivary glands. S3/UIS4ko sporozoites also infect the liver of experimental mice.

However, we surprisingly discovered that the S3/UIS4ko parasites are defective in complete liver stage development and thus unable to initiate subsequent blood stage infection. Analysis of infection in the liver 48 hrs after inoculation of 25,000 S3/UIS4ko sporozoites into eight mice by reverse transcriptase (RT)-PCR using oligonucleotide primers to the parasite 18S ribosomal RNA (Bruna-Romero *et al.*, 2001) showed that liver stages were undetectable at that time point in all eight mice (Figure 2). Five control mice each infected with 25,000 wildtype (wt) sporozoites showed liver stages at the same time point using the same RT-PCR protocol (Figure 2).

Furthermore, ten mice each infected with 10,000 S3/UIS4ko sporozoites never developed a patent blood stage infection as monitored by microscopic examination of Giemsa-stained thin blood smears over a period of 30 days. 5 control mice each injected with 10,000 wt sporozoites developed a patent blood stage infection after 3 days.

Therefore, S3/UIS4ko sporozoites are attenuated and can be used to immunize mice without causing a blood stage infection. Four mice were immunized three times with S3/UIS4ko sporozoites (doses were 21,000, 20,000 and 10,000 sporozoites) and were then inoculated with 20,000 infectious wt sporozoites (challenge). The mice did not develop a blood stage infection as determined by thin blood smear over a period of 30 days, showing that the immunization with S3/UIS4ko sporozoites had protected the animals against wt liver stage development and thus initiation of blood stage infection (sterile protection). Eight non-immunized control animals inoculated with the same number of infectious wt sporozoites developed blood stage infection after three days.

We have identified the gene corresponding to S3/UIS4 in the human malaria parasite *P. falciparum* (accession number: NP_700638, PF10_0164). Thus, we expect that disruption of this gene will create a *P. falciparum* sporozoite that can be used to immunize and protect humans against malaria infection by mosquito bite. A genetically attenuated parasite defective in a defined gene would be preferable over radiation-attenuated sporozoites as an immunogen for reasons described above.

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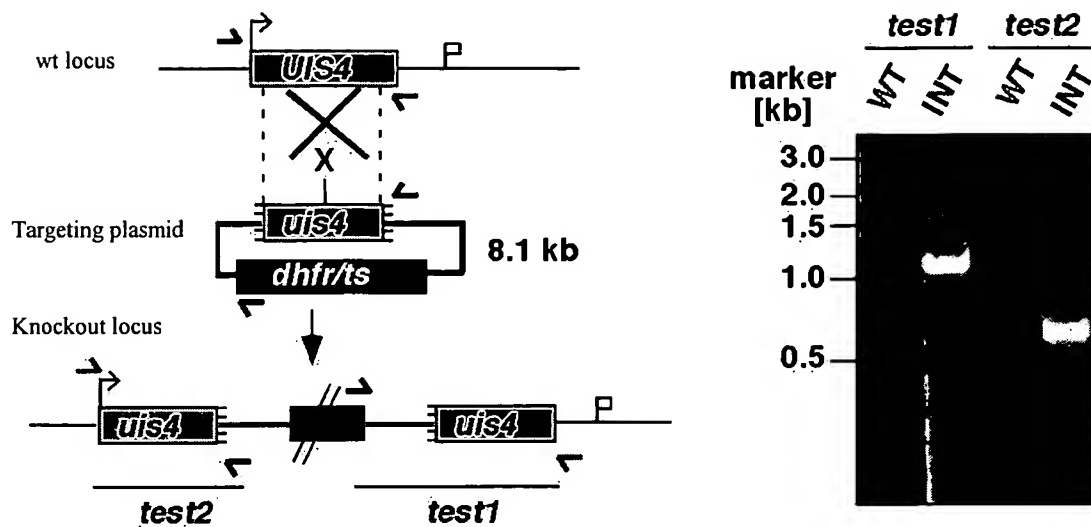


Figure 1. Cartoon depicting the knockout strategy that was used to inactivate the S3/UIS4 gene (UIS4 in the cartoon). The targeting plasmid consists of a 5' and 3' truncated version of S3/UIS4 and a drug-selectable marker (*dhfr/ts*). The right panel shows test PCRs on obtained knockout clones after transfection with primers at positions shown in the cartoon. PCR demonstrates that the targeting plasmid had integrated (INT) into the wildtype (wt) locus.

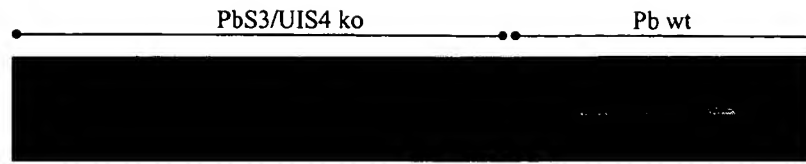


Figure 2. RT-PCR shows that S3/UIS4ko parasites are unable to develop late liver stages. RT-PCR with oligonucleotide primers to the *P. berghei* (Pb) parasites 18S ribosomal RNA and RNA template extracted from the livers of infected mice 48 hours after inoculation of 25,000 sporozoites. The left eight lanes show no amplification from eight mice infected with PbS3/UIS4ko sporozoites and the right 5 lanes show positive amplification from 5 mice infected with wildtype (wt) sporozoites.

Differential transcriptome profiling identifies *Plasmodium* genes encoding pre-erythrocytic stage-specific proteins

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Summary

Invasive sporozoite and merozoite stages of malaria parasites that infect mammals enter and subsequently reside in hepatocytes and red blood cells respectively. Each invasive stage may exhibit unique adaptations that allow it to interact with and survive in its distinct host cell environment, and these adaptations are likely to be controlled by differential gene expression. We used suppression subtractive hybridization (SSH) of *Plasmodium yoelii* salivary gland sporozoites versus merozoites to identify stage-specific pre-erythrocytic transcripts. Sequencing of the SSH library and matching the cDNA sequences to the *P. yoelii* genome yielded 25 redundantly tagged genes including the only two previously characterized sporozoite-specific genes encoding the circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP). Twelve novel genes encode predicted proteins with signal peptides, indicating that they enter the secretory pathway of the sporozoite. We show that one novel protein bearing a thrombospondin type 1 repeat (TSR) exhibits an expression pattern that suggests localization in the sporozoite secretory rhoptry organelles. In addition, we identified a group of four genes encoding putative low-molecular-mass proteins. Two proteins in this group exhibit an expression pattern similar to TRAP, and thus possibly localize in the sporozoite secretory micronemes. Proteins encoded by the differentially expressed genes identified here probably mediate

specific interactions of the sporozoite with the mosquito vector salivary glands or the mammalian host hepatocyte and are not used during merozoite–red blood cell interactions.

Introduction

Protozoa belonging to the phylum Apicomplexa are obligate intracellular parasites and therefore entirely dependent on successful recognition and invasion of a suitable host cell to achieve propagation. Host cell invasion is the task of highly specialized, invasive stages that attach to the host cell plasma membrane (PM) and generally enter by active penetration powered by a unique parasite actomyosin motor (Heintzelman, 2003). Concurrent with entry, a membrane-bound compartment (parasitophorous vacuole, PV) derived from the host cell PM forms around the parasite (Lingelbach and Joiner, 1998). The PV separates the intracellular parasite from the host cell cytoplasm and provides a sheltered environment for further development. Invasive stages harbour several unique secretory organelles (apical organelles) named micronemes, rhoptries and dense granules (Aikawa and Sterling, 1974), each containing a complex assortment of proteins (Caruthers, 1999; Preiser *et al.*, 2000). After an invasive stage initiates contact with a suitable host cell, an interaction that involves parasite surface ligands, the apical organelles sequentially discharge and release proteins that are instrumental in mediating the invasion process and the subsequent establishment of the intracellular phase.

The malaria parasite *Plasmodium* uses three distinct, highly specialized invasive stages, the merozoite, the sporozoite and the ookinete, each adapted to interact with specific host tissues within the mosquito vector or vertebrate host. Merozoites, the invasive stage that enters red blood cells, are most frequently studied, mainly because blood stages can be obtained in sufficient quantity from continuous culture or animal infections (Blackman and Bannister, 2001). Therefore, this stage is amenable to routine biochemical and molecular biological analysis. Consequently, the biology and composition of the merozoite surface and its apical organelles are best understood, and most proteins have been characterized in this

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stage (Preiser *et al.*, 2000). In contrast, little is known about the surface and the apical organelles of salivary gland sporozoites (Kappe *et al.*, 2003), the invasive parasite stage that is transmitted by mosquito bite and first establishes the mammalian infection with its entry into hepatocytes. Although the principal mechanisms of host cell invasion are probably shared between sporozoites and merozoites, it is considered likely that the set of surface proteins and apical organelle proteins expressed by each invasive stage differ significantly, reflecting the need to interact with entirely different host tissues. Expression of proteins that are required for recognition and invasion of hepatocytes is exemplified by the circumsporozoite protein (CSP), a sporozoite surface protein, and the thrombospondin-related anonymous protein (TRAP), a sporozoite micronemal protein (Menard, 2000), the only sporozoite-specific invasion-related proteins identified to date.

We hypothesized that, by analysing differences in transcript profiles of sporozoites and merozoites, it should be possible to identify sporozoite-specific transcripts, some of which may encode novel proteins of the sporozoite surface or the apical organelles.

Results

Identification of *Plasmodium* sporozoite-expressed genes that are not expressed in blood stages

Hepatocytes and red blood cells are dramatically different types of host cells. Differential host cell interaction by salivary gland sporozoites and merozoites may be reflected by major expression differences of secreted and membrane-associated parasite ligands, whereas components of the intracellular motor machinery are probably shared among invasive stages. Therefore, we evaluated transcriptome differences of sporozoites and merozoites. Using 5.2×10^5 highly purified *Plasmodium yoelii* sporozoites, we obtained ≈ 25 ng of poly(A)⁺ RNA. We performed a suppression subtractive hybridization screen (SSH), a cDNA-based screening technique that allows simultaneous normalization and subtraction of cDNA populations with low amounts of starting material (Diatchenko *et al.*, 1999). To enrich for sporozoite-specific transcripts, we used the sporozoite cDNA as our tester and a 100-fold excess of cDNA obtained from a schizont-enriched blood-stage parasite fraction combined with uninfected salivary glands as our driver. We hypothesized that, using this approach, we could minimize profiling of housekeeping gene expression in sporozoites that is shared with merozoites, as well as contaminating mosquito sequences.

We obtained and sequenced 500 cDNA clones and matched their sequences to the *P. yoelii* genome

sequence (Carlton *et al.*, 2002). None of the sequenced clones was of mosquito origin. Twenty-five individual genome contigs were tagged by at least two cDNA clones, and we pursued further only these redundantly tagged genes to avoid an increased incidence of false positives. Notably, the SSH screen did not tag any of the known components of the *Plasmodium* invasion motor machinery, i.e. *MyoA* (Pinder *et al.*, 1998; Matuschewski *et al.*, 2001), *MTIP* (Bergman *et al.*, 2003) and *MCP-1* (Klotz *et al.*, 1989; Matuschewski *et al.*, 2002a). These proteins are shared among sporozoites and merozoites. To confirm whether the redundantly tagged genes are specifically expressed in sporozoites, we probed dot-blots of representative cDNAs for each gene with labelled amplified total cDNA probes from either salivary gland sporozoites or mixed blood stages (Fig. 1A). As a quality control for the blood-stage cDNA probe, we added a cDNA clone encoding the merozoite surface protein-1 (MSP-1) (Lewis, 1989). Transcripts were detected for all 25 genes in salivary gland sporozoites, but no transcripts were detected in mixed blood stages. We thus named the genes S genes (S for sporozoite). Our approach was validated by the presence of *CSP* (Nussenzweig and Nussenzweig, 1989) and *TRAP/SSP2* (Rogers *et al.*, 1992) that were among the most abundant S genes, corresponding to S2 and S8 respectively (Fig. 2). Of the remaining S genes, two transcripts, *S3/UIS4* and *S7/UIS3*, had been isolated previously in a screen designed to enrich for genes that are specifically upregulated in mature, infective salivary gland sporozoites and absent from oocyst sporozoites (Matuschewski *et al.*, 2002a). We analysed further expression of selected S genes by reverse transcriptase polymerase chain reaction (RT-PCR) using total RNA from sporozoites or mixed blood stages as templates. Expression in sporozoites was detected for all tested S genes, but no expression could be detected in mixed blood stages (Fig. 1B). BLASTP search of the predicted proteins of *Plasmodium falciparum* (Gardner *et al.*, 2002) deposited in GenBank and PlasmoDB identified putative orthologues for all *P. yoelii* S genes except S3 and S12 (Fig. 2). The identification of *P. falciparum* S gene orthologues allowed us to compare our expression results with a recently published microarray expression analysis of *P. falciparum* blood stages and sporozoites (Le Roch *et al.*, 2003) (Fig. 2). Most of the *P. falciparum* S gene orthologues were highly induced in sporozoites compared with mixed asexual stages (ranging from ≈ 620 -fold induction in the case of *S21/TRSP* to ≈ 40 -fold induction for S1 and S2/*CSP*). S15 and S20 showed low, but still significant, induction of four- and threefold respectively. Some *P. falciparum* S genes (S6, S9, S11, S17 and S18) were expressed at low levels in blood stages and sporozoites. Our SSH screen possibly identified these S genes as differentially expressed because it included a normalization step, thus allowing for

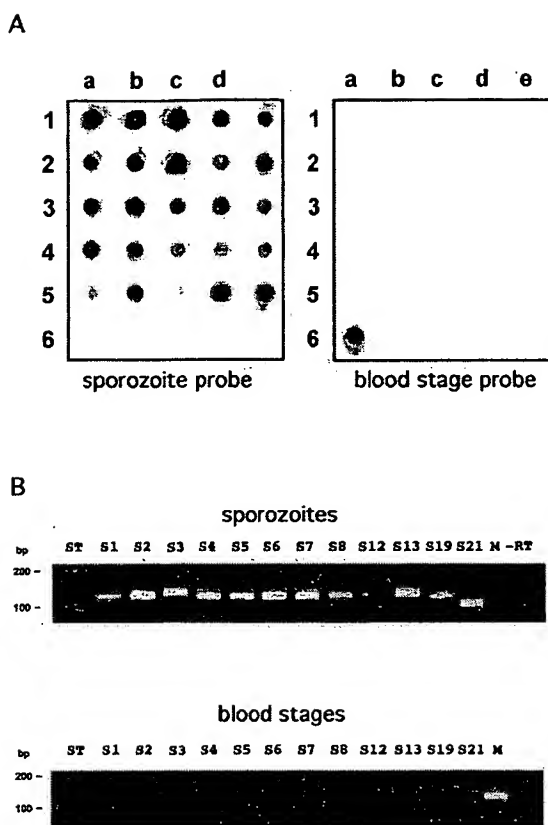


Fig. 1. Identification of *Plasmodium* sporozoite-restricted genes (S genes). Twenty-five genes were redundantly recovered in a forward suppression subtractive hybridization screen designed to enrich for sporozoite-specific transcripts that are absent in blood stages. **A.** To verify stage-specific expression of the S genes, cDNA dot-blot (a1 to e5) were hybridized with labelled total cDNA probes generated from *P. yoelii* salivary gland sporozoites (left) and mixed blood stages (right). As a control for the quality of the blood-stage cDNA, a cDNA fragment encoding the major merozoite surface protein PymSP-1 was added (a6). **B.** Reverse transcriptase-PCR was used to verify further differential expression of selected S genes. PCR products of the expected sizes were amplified for all tested S genes from salivary gland sporozoite total RNA (top) but not from mixed blood-stage total RNA (bottom). A negative control reaction without RT was included for genomic DNA contamination using the S2 primers (-RT), and a primer pair specific for MSP-1 (M) was used as a positive control for the blood-stage RNA. Sizes of the DNA standards (ST) are given in basepairs (bp).

differential detection of rare transcripts. In contrast to our analysis that detected *P. yoelii* S19 expression only in sporozoites (Fig. 1), the *P. falciparum* orthologue of S19 was equally detected in sporozoites and blood stages by microarray analysis.

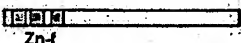

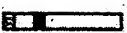



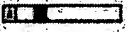


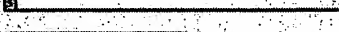
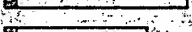

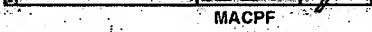

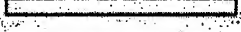


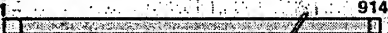
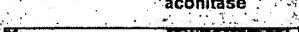

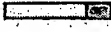

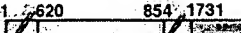
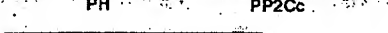
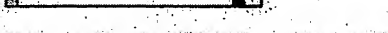
S1 encodes a novel putative *Plasmodium* sporozoite C3H1 zinc finger protein

The 23 novel S genes have not been studied previously

and may provide important leads towards understanding stage-specific gene regulation, signal transduction as well as host cell recognition. The S1 gene encodes a 409-amino-acid protein that contains three C3H1-type zinc fingers in its amino-terminal portion. Proteins containing zinc finger domains of the C-x8-C-x5-C-x3-Hs type are considered to be important regulatory proteins that exert their function through direct binding of the 3' untranslated region of various mRNAs. For instance, it has been shown that tristetraprolin (TTP), the prototype of a class of C3H1 zinc finger proteins, binds directly to the AU-rich elements of the tumour necrosis factor (TNF) α mRNA, resulting in its destabilization and, hence, inhibition of TNF α production (Carballo *et al.*, 1998; Lai *et al.*, 1999). No function has been assigned to the S1 protein yet (Gardner *et al.*, 2002); however, the presence of three C3H1 zinc fingers indicates a role for S1 in post-transcriptional control of gene expression that is specifically required in sporozoites.

Novel candidates for *Plasmodium* sporozoite-specific host cell interaction

S2/CSP and S8/TRAP/SSP2 are essential for sporozoite development, sporozoite motility as well as host cell recognition and entry (Menard *et al.*, 1997; Sultan *et al.*, 1997). Both contain adhesion domains that confer specific host cell binding. Whereas CSP is probably linked to the PM via a glycosylphosphatidylinositol anchor, TRAP/SSP2 is a type 1 transmembrane protein that can transmit an extracellular recognition event to the parasite's interior. Like CSP and TRAP, 12 of the 23 novel S proteins contain putative N-terminal signal sequences, suggesting that they are targeted to the secretory pathway and may become surface exposed, either constitutively or by inducible secretion through the apical organelles. The S6 gene encodes an intriguing 2689-amino-acid protein. It contains a transmembrane domain and a 47-amino-acid cytoplasmic domain in its C-terminal portion that is typically found in members of the apicomplexan TRAP family (Fig. 3) (Menard, 2001). The defining features of this domain are a penultimate tryptophan residue together with multiple negatively charged residues that are vital for sporozoite motility and invasion (Kappe *et al.*, 1999). Recently, it was shown that the TRAP family cytoplasmic domains interact with aldolase, which in turn interacts with actin (Jewett and Sibley, 2003). In its putative extracellular portion, S6 contains highly repetitive sequence elements and a 1424-amino-acid region with homologies to immunoglobulin-like domains (*e*-value $9e-46$). The presence of this domain, which is associated with protein-protein and protein-ligand interactions, in a sporozoite-restricted protein implies its involvement in a specific recognition event, possibly during hepatocyte invasion.

S gene	cDNA clone#	clone (Fig. 1)	gene product	10 kDa	<i>P. falciparum</i> ortholog			
					Acc. #	locus	aa ident.	microarray induction
S1	72	1a	 Zn-f		EAA17779	PF10_0083	39%	39x
S2/CSP	33	1b	 RI RII TSR GPI		AAA29558	PFC0210c	27%	40x
S3/UIS4	24	1c			EAA21410	-	-	-
S4	22	1d			AY427752	PFL0800c	44%	60x
S5	18	1e		999	EAA16050	PFD0425w	54%	120x
S6	13	2a	 Ig-like CTD	1 673 1285 2720	EAA16943/ BK001658	PF14_0404	22%	low expr.
S7/UIS3	14	2b			EAA22537	PF13_0012	31%	110x
S8/SSP2/TRAP	11	2c	 A-domain TSR CTD	826	Q01443	PF13_0201	30%	335x
S9	11	2d			EAA20893	PF14_0168	37%	low expr.
S10	10	2e			EAA20040/ BK001660	PFD0235c	26%	79x
S11	9	3a			EAA19048	PF14_0044	51%	low expr.
S12	8	3b			EAA22575	-	-	-
S13/PPLP1	7	3c	 MACPF	815	EAA16049/ BK001661	PFD0430c	57%	130x
S14	7	3d			EAA15981	PFL0370w	43%	83x
S15	7	3e			EAA17348/ BK001662	PF08_0073	57%	4x
S16	7	4a	 AAA-ATPase		EAA18580	MAL6P1.181	25%	N/D
S17	6	4b			EAA22739	PFB0425c	32%	low expr.
S18	6	4c	 aconitase	914	EAA22713	PF13_0229	78%	low expr.
S19	4	4d	 thioredoxin-like		EAA17343	PFI0950w	56%	none
S20	4	4e	 Kelch		EAA18035	PFL0650c	63%	3x
S21/TRSP	3	5a	 TSR		EAA19449/ BK001663	PFA0200w	43%	620x
S22	2	5b	 PH PP2Cc	1 620 854 1731 2017 3063	EAA15711/ BK001664	PF11_0480	25%	340x
S23	2	5c			EAA21163/ BK001665	PF08_0088	47%	138x
S24	2	5d			EAA18498/ BK001666	PFA0205w	32%	26x
S25	2	5e	 KISc	1688	EAA21870	PFL0545w	50%	45x

Another protein of interest is S13, a putative secreted protein bearing a domain with significant similarity to membrane attack complex/perforin (MACPF) domains (e-value: 1.5×10^{-9}). This domain is present in pore-forming proteins of mammalian complement system and perforin (Liu *et al.*, 1995; Morgan, 1999). We have shown recently

that this *Plasmodium* perforin-like protein (PPLP1) localizes to the secretory micronemes of salivary gland sporozoites (Kaiser *et al.*, 2003).

The S21 gene encodes a novel protein that we named TRSP (thrombospondin-related sporozoite protein). The complete open reading frame (ORF) was determined by

Fig. 2. Schematic diagram of the predicted primary structure of the *P. yoelii* sporozoite-specific (S) gene products. Putative cleavable signal peptides are indicated by red boxes, putative transmembrane domains are shown as blue boxes, and repeat regions as striped areas. Putative domains with assigned functions are represented by coloured boxes. For each S gene, the number of clones identified in the suppression subtractive hybridization screen, the respective GenBank™ accession number of the *P. yoelii* gene and the corresponding *P. falciparum* orthologues with GenBank™ accession number and the percentage of amino acid identities are shown. The values for microarray induction of *P. falciparum* S gene orthologues were calculated based on published data (Le Roch *et al.*, 2003). For each *P. falciparum* S orthologue, the ratio of sporozoite expression and the mean expression of asexual stages generated by two different synchronization methods is displayed as 'fold induction'. Sporozoite expression levels below 30 were set as low expression, because similar expression levels in blood stages are often accompanied by relatively large variations among the two synchronization methods. A-domain, von Willebrand factor A-domain; CTD, TRAP-family cytoplasmic tail domain; GPI, glycosylphosphatidylinositol anchor; Ig-like, immunoglobulin-like; KISc, kinesin motor catalytic domain; MACPF, membrane attack complex/perforin; PH, pleckstrin homology domain; R1, region I; RIII, region III; TSR, thrombospondin type I repeat; Zn-f, zinc finger.

comparison of cDNA obtained from sporozoites and genomic DNA. This analysis revealed two introns and a spliced ORF encoding a 174-amino-acid protein with a predicted molecular mass of ≈ 18 kDa. The predicted protein has a putative N-terminal cleavable signal peptide and a carboxyl-terminal hydrophobic region that could serve as a membrane anchor (Fig. 4A). The *P. yoelii* and *P. falciparum* genes have an identical exon/intron structure, and the deduced proteins share 43% amino acid sequence identity (Fig. 4A). We also noted the conservation of the short cytoplasmic tail (IKKKI) between the two species. Both proteins bear a thrombospondin type 1 repeat (TSR) domain (*e*-value $3.9\text{e-}11$). TSR domains are adhesive modules that bind to sulphated glycoconjugates. They are found in a variety of metazoan proteins such as thrombospondin, properdin, complement components and neural adhesion glycoproteins (Adams and Tucker, 2000). In *Plasmodium* species, TSRs are found in CSP, TRAP and in CS protein TRAP-related protein (CTRP) (Trottein *et al.*, 1995). Hybridization of *P. yoelii* salivary gland sporozoite cDNA and mixed blood-stage cDNA with a *S21/TRSP*-specific probe confirmed expression in sporozoites and the lack of expression in blood stages (Fig. 4B). To investigate transcript levels of *S21/TRSP* in the transition from oocyst sporozoites to salivary gland sporozoites, we used quantitative real-time reverse transcriptase PCR (QRRT-PCR) using gene-specific oligonucleotide primers and total RNA isolated from the two *P. yoelii* sporozoite populations as a template. The QRRT-PCR detected *S21/TRSP* transcripts in oocyst sporozoites and salivary gland sporozoites with slightly higher transcript levels in salivary gland sporozoites (Fig. 4C).

Immunoblot analysis using polyclonal rabbit antisera raised against a recombinant *S21/TRSP*-GST fusion protein and total protein isolated from *P. yoelii* oocyst sporozoites and salivary gland sporozoites revealed two bands of ≈ 20 kDa and ≈ 10 kDa apparent molecular mass in each sporozoite population (Fig. 4D). The higher molecular mass band may correspond to the full-length protein, which has a predicted mass of 18 kDa. The 10 kDa species is possibly a cleaved N-terminal fragment of *S21/TRSP*. The antiserum reacted with *P. yoelii* salivary gland sporozoites and oocyst sporozoites in indirect immunofluorescence assay (IFA), resulting in a unique, polarized staining pattern at one end of the sporozoite (Fig. 5). Dual IFA with the sporozoite micronemal protein TRAP, using salivary gland sporozoites, revealed that the TRSP fluorescent signal is distinct and does not overlap significantly with the TRAP signal (Fig. 5A). This suggests that TRSP does not localize to the micronemes. Dual IFA with CSP in oocyst sporozoites showed that TRSP localization is also distinct from the localization of this surface protein (Fig. 5B). The fluorescent pattern of TRSP instead revealed an elongated structure, sometimes appearing bilobed, that extends from the sporozoite mid-portion to one extreme end. This fluorescent pattern may indicate a localization of TRSP to the rhoptries, paired elongated organelles in sporozoites that extend from the mid-portion of the parasite to the apical end where both rhoptry lobes converge on the apical prominence (Aikawa and Sterling, 1974). Unfortunately, no sporozoite rhoptry proteins have been identified thus far, precluding co-localization studies.

Four single-exon genes, *S3*, *S4*, *S7* and *S12*, encode low-molecular-mass proteins each carrying a putative

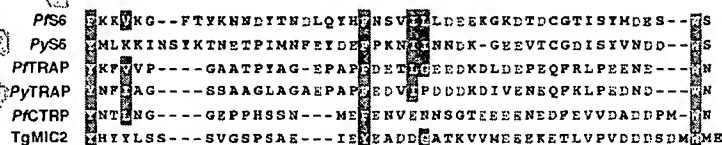


Fig. 3. S6 has a TRAP family-like cytoplasmic domain. An amino acid sequence alignment of the putative cytoplasmic domains of *P. falciparum* S6 (PfS6) and *P. yoelii* S6 (PyS6) and the cytoplasmic domains of *P. falciparum* TRAP (PtTRAP), *P. yoelii* TRAP (PyTRAP), *P. falciparum* CTRP and *Toxoplasma gondii* MIC2 (TgMIC2) are shown. TRAP-like cytoplasmic domains show little overall amino acid sequence conservation but share a functionally important penultimate tryptophan residue and acidic residues. These features are conserved in S6. Conserved residues are shown in white with grey background. The acidic residues are shown in grey, and all other residues are shown in black.

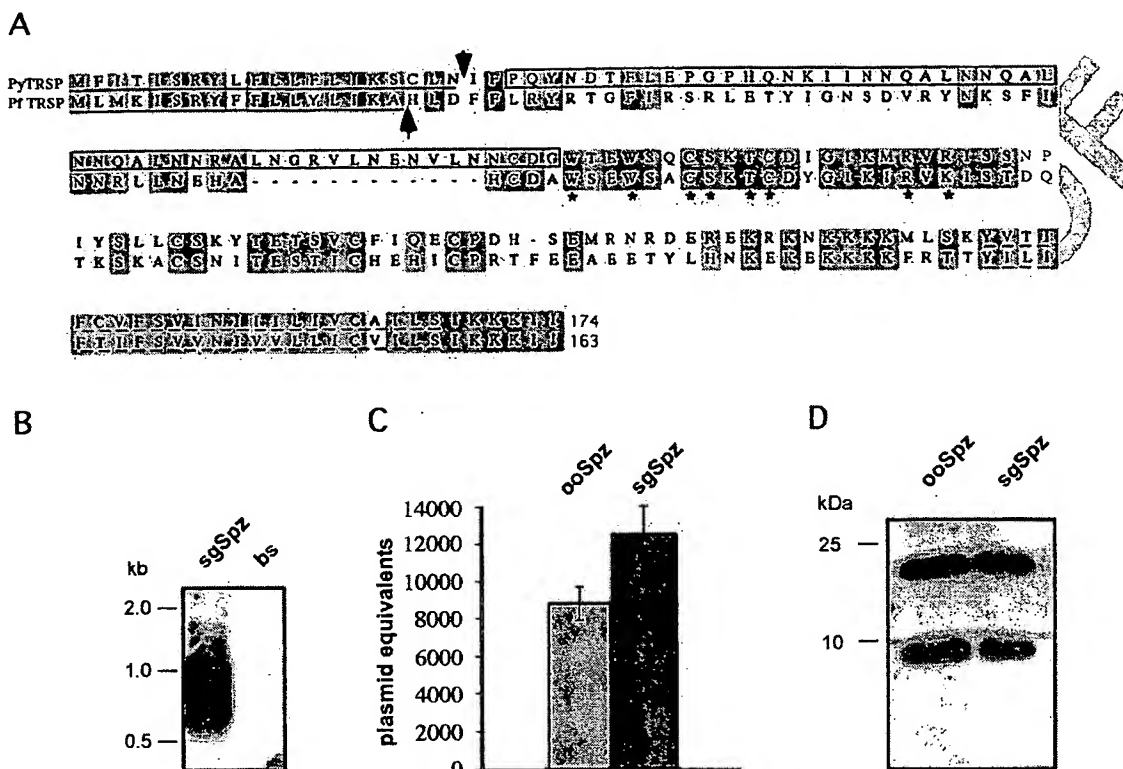


Fig. 4. S21/TRSP is conserved among *Plasmodium* species.

A. Amino acid sequence alignment of *P. yoelii* TRSP (PyTRSP) and *P. falciparum* TRSP (PtTRSP). Identical residues are shaded dark grey. Conserved amino acid changes are shaded light grey, and radical changes are not shaded. The putative signal peptide is underlined with a solid line, and the cleavage sites are marked with arrowheads. Potential membrane-spanning regions are underlined with a dashed line. Conserved residues of the thrombospondin type 1 repeat (TSR) domain are marked by asterisks (*). The amino acid segment of PyTRSP expressed as a GST fusion protein is marked with a solid box.

B. cDNA blot of mixed blood stages and salivary gland sporozoites of *P. yoelii* hybridized to a labelled TRSP probe. Transcripts are abundant in sporozoites (sgSpz), but no transcripts are detected in mixed blood stages (bs).

C. Quantitative real-time reverse transcriptase (QRRT)-PCR shows similar TRSP expression in oocyst sporozoites (ooSpz) and salivary gland sporozoites (sgSpz). Transcript quantity is represented as the number of copies (\pm SD) in comparison with an external standard curve generated with gene-specific plasmids. Experiment was done in triplicate.

D. Immunoblot analysis of oocyst sporozoite (ooSpz) and salivary gland sporozoite (sgSpz) extracts. The anti-PyTRSP antiserum specifically recognized two protein species that migrated at \sim 20 kDa and \sim 10 kDa in both sporozoite extracts.

N-terminal cleavable signal peptide. In addition, S3 and S7 harbour an internal segment of hydrophobic amino acids that could function as a transmembrane domain. Orthologous *P. falciparum* proteins were identified for S4 and S7 but not for S3 and S12. QRRT-PCR detected very low transcript levels for S3 and S7 in oocyst sporozoites but high transcript levels in salivary gland sporozoites, corresponding to a 200-fold increase in transcript abundance for S3 and a 25-fold increase for S7 in salivary gland sporozoites (Fig. 6A and B). A control QRRT-PCR for TRAP detected high levels of TRAP transcript in both sporozoite populations (Fig. 6C). Hybridization of salivary gland sporozoite cDNA and mixed blood-stage cDNA with S3- and S7-specific probes confirmed their expression in sporozoites and their lack of expression in blood

stages (Fig. 6D and E). Similar expression patterns were observed for S4 and S12 (data not shown). Immunoblot analysis of total protein isolated from oocyst sporozoites, salivary gland sporozoites and mixed blood stages using polyclonal rabbit antisera raised against a recombinant S3-GST fusion protein revealed a single band of \sim 30 kDa apparent molecular mass in salivary gland sporozoites. No reactivity was observed in oocyst sporozoites and mixed blood stages (Fig. 6F). The slow migration of S3 (predicted mass of 23 kDa) in SDS-PAGE gels may be caused by the protein's high and unevenly distributed charge. Polyclonal mouse antisera raised against a recombinant S7-GST fusion protein did not react with parasite extracts in immunoblot analysis. We examined the localization of S3 and S7 in sporozoites by IFA

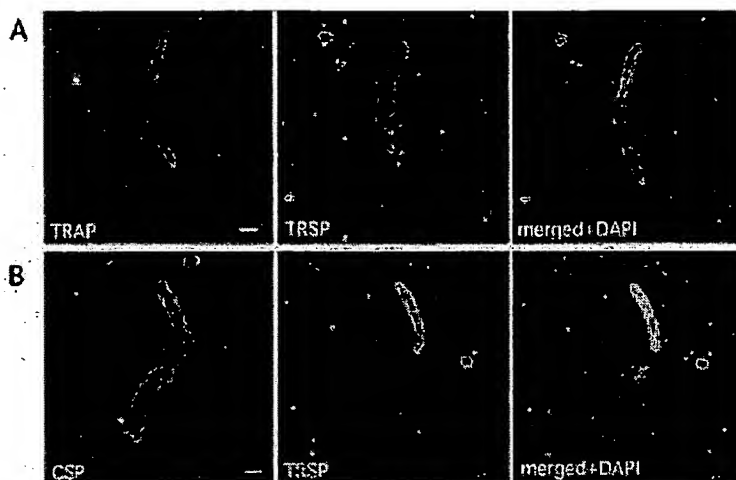


Fig. 5. Localization of *P. yoelii* TRSP in sporozoites.

A. Dual indirect immunofluorescence assay (IFA) on salivary gland sporozoites with antiserum specific for PyTRAP, a sporozoite micronemal protein, and with antiserum specific for PyTRSP. TRAP staining is internal and not restricted to one pole of the sporozoite. TRSP has a different distribution, showing an internal, bilobed staining pattern that extends from the nuclear region to the apical end of the sporozoite where the two lobes seemingly converge. B. Dual IFA on oocyst sporozoites with antiserum specific for PyCSP, a sporozoite surface protein, and with antiserum specific for PyTRSP. TRSP localization is distinct from the surface-localized CSP. The staining patterns are most consistent with TRSP localization to the paired rhoptry organelles. The sporozoite nucleus was visualized with 4',6-diamidino-2-phenylindole (DAPI). Scale bar is 1 μ m.

(Fig. 7). The S3 and S7 staining of salivary gland sporozoites appeared to be internal and granular, with no staining observed in the nuclear region. Co-localization of S3 with TRAP, which has previously been localized to the micronemes, showed extensive overlap of the fluorescent signals (Fig. 7A). Co-localization of S3 with S7 showed similar expression patterns with extensive but not complete overlap (Fig. 7B). The S3 and S7 IFA patterns are different from the IFA pattern obtained for TRSP (Fig. 5).

Discussion

We have used differential transcriptome profiling to identify genes that are expressed in the *Plasmodium* sporozoite stage but not in the merozoite or other blood stages of the parasite. Somewhat surprisingly, 12 out of the 23 identified differentially expressed novel genes encode predicted secreted or membrane-bound proteins that are probably positioned in the sporozoite apical organelles or on the sporozoite surface. Although the SSH screen is not

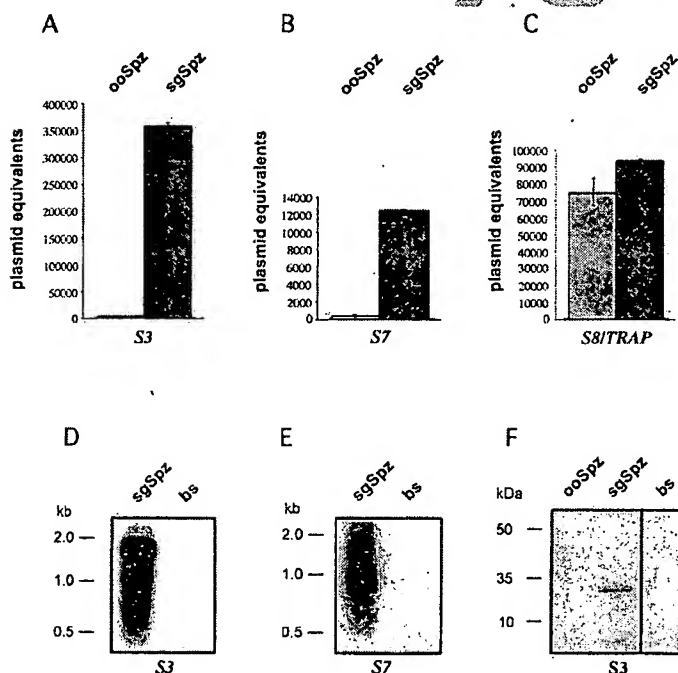


Fig. 6. Stage-specific expression of PyS3 and PyS7.

A and B. Quantitative real-time reverse transcriptase (QRT)-PCR analysis shows significantly different transcript levels for S3 and S7 in oocyst sporozoites (ooSpz) and salivary gland sporozoites (sgSpz). Transcript levels in salivary gland sporozoites are ≈ 200 -fold higher for S3 and ≈ 25 -fold higher for S7 compared with oocyst sporozoites. C. Control QRT-PCR amplification for TRAP shows similar transcript levels among sporozoites. Transcript quantity is represented as the number of copies (\pm SD) in comparison with an external standard curve generated with gene-specific plasmids. Experiment was done in triplicate.

D and E. cDNA blots of mixed blood stages and salivary gland sporozoites of *P. yoelii* hybridized to a labelled S3 probe (D) and S7 probe (E). Transcripts for S3 and S7 are abundant in salivary gland sporozoites (sgSpz), but no transcripts are detected in mixed blood stages (bs). F. Immunoblot analysis of oocyst sporozoite (ooSpz), salivary gland sporozoite (sgSpz) extracts and mixed blood-stage (bs) extracts with the anti-PyS3 antiserum. A protein species that migrated at ≈ 30 kDa is only detected in salivary gland sporozoite extracts but not in oocyst sporozoite and mixed blood-stage extracts.

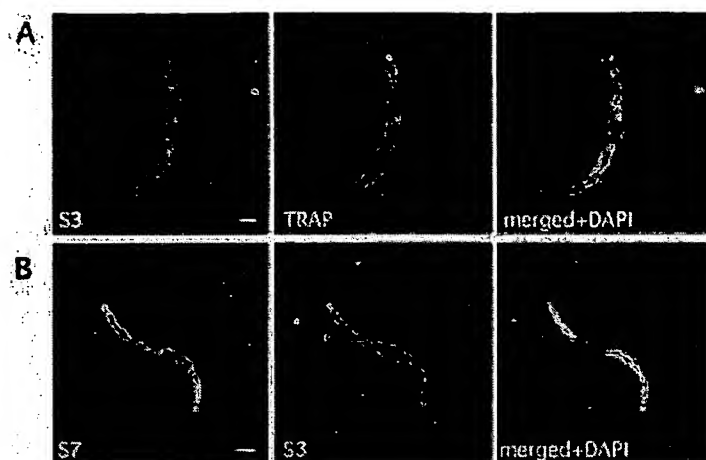


Fig. 7. Localization of *P. yoelii* S3 and S7 in salivary gland sporozoites.

A. Dual IFA with antiserum specific for PyS3 and antiserum specific for PyTRAP. The merged fluorescent image reveals highly similar S3 and TRAP staining patterns, both showing internal, granular localization that is not restricted to one pole of the sporozoite and excluded from the nuclear region.

B. Dual IFA with antiserum specific for PyS3 and antiserum for PyS7. The merged fluorescent image reveals extensive but not complete overlap of the S3 and S7 signals. Like S3, S7 shows internal, granular localization that is not restricted to one pole of the sporozoite and excluded from the nuclear region. The sporozoite nuclei were visualized with DAPI. Scale bar is 1 µm.

exhaustive for the identification of differentially expressed genes, it appears that a significant proportion of sporozoite transcripts encode secreted or membrane-bound proteins that are not expressed in merozoites. We investigated the potential apical organelle localization for three of these proteins. S21/TRSP showed a staining pattern that was consistent with a possible localization to the rhoptries. S3 and S7 showed a similar expression pattern when compared with TRAP, suggesting that both may be micronemal proteins. We have also recently investigated the localization of S13/PPLP1 by IFA and immunoelectron microscopy, showing that it is a sporozoite-specific micronemal protein (Kaiser *et al.*, 2003). Previously, the similarity of the protein composition of the apical organelles and the surface of sporozoites and merozoites had not been well addressed. Numerous merozoite surface proteins and proteins of the apical complex have been identified (Preiser *et al.*, 2000; Berzins, 2002), some of which have also been shown to be expressed in sporozoites using available antibodies (Gruner *et al.*, 2001; Kappe *et al.*, 2001; Preiser *et al.*, 2002). A recent proteomic analysis of *P. falciparum* sporozoites (Florens *et al.*, 2002) identified peptides matching various merozoite apical organelle and surface proteins, indicating that the two stages may have a large number of invasion-related proteins in common. Conversely, the two thoroughly characterized invasion-related sporozoite proteins, the surface protein CSP and the micronemal protein TRAP, are sporozoite specific. Clearly, sporozoites need unique surface and apical organelle proteins to interact specifically with the mosquito salivary glands and mammalian hepatocytes but not red blood cells, which they encounter frequently in the bloodstream after inoculation by mosquito bite. Our study substantially expands the set of sporozoite-specific putative apical organelle proteins, which up to now consisted solely of TRAP. Some of the

novel proteins carry domains that are likely to be involved in sporozoite-salivary gland and sporozoite-hepatocyte interactions. For example, TRSP is the fourth *Plasmodium* protein identified that contains a consensus TSR (Adams and Tucker, 2000). TSR motifs are also present in CSP and TRAP, each carrying a single TSR, and in the ookinete-expressed protein CTRP, which carries seven TSRs (Menard, 2001). TSRs mediate binding to sulphated glycoconjugates. The consensus-conserved motifs of TSRs are the amino-terminal tetrapeptide WXXW that can act as a heparin-binding motif (Guo *et al.*, 1992a,b) and a carboxyl-terminal cluster of basic residues. This cluster, which is also present in the TSR of CSP, was shown to bind sulphated glycoconjugates (Sinnis *et al.*, 1994; Ganitt *et al.*, 1997). In addition, a central motif in the TSR of thrombospondin (CSVTG) was described as a cell adhesion motif (Tuszynski *et al.*, 1992). A study using sporozoites with introduced mutations in the TSR of TRAP indicated that this module facilitates sporozoite entry into the mosquito salivary glands and hepatocytes to a similar extent (Matuschewski *et al.*, 2002b). Based on the exquisite conservation of the TSR module in TRSP, it is likely that this protein has adhesive properties. As TRSP is expressed in oocyst sporozoites and salivary gland sporozoites, it might act in concert with TRAP or CSP during sporozoite-host cell interactions in the mosquito vector and the mammalian host, a possibility that surely warrants further investigation.

PfS7 was described previously as a partial open reading frame (ORF; *orfP*) that had a subtelomeric location on chromosome 13 of *P. falciparum* (Sallicandro *et al.*, 2000). The region of chromosome 13 containing *PfS7* was shown to be frequently deleted in laboratory lines of *P. falciparum* that were maintained in *in vitro* blood stage culture but this deletion was not found in *P. falciparum* field isolates (Sallicandro *et al.*, 2000). These data suggested that the

region was maintained by selective pressure exerted on the parasites' pre-erythrocytic stages that undergo natural transmission through the mosquito vector. We have shown here that PyS7 is indeed a sporozoite-expressed protein and that it is not expressed in blood stages. Furthermore, we showed previously that S7 and S3 transcript expression was upregulated when sporozoites entered the mosquito salivary glands (Matuschewski *et al.*, 2002a), and we confirmed the expression patterns in this study using QRRT-PCR. Thus, S7 and S3 may be important for the initial infection of the mammalian liver by the sporozoite stage. Recently, a family of blood stage-expressed, low-molecular-mass proteins named early transcribed membrane proteins (ETRAMPs) was characterized in *P. falciparum* (Spielmann *et al.*, 2003). The study placed PfS7 as ETRAMP13 within this family. In accordance with our results, no blood-stage expression of transcripts was detected for S7/ETRAMP13. Interestingly, blood stage-expressed ETRAMPs localized to the parasitophorous vacuole membrane (PVM) of intraerythrocytic stages and formed distinct PVM domains within the membrane when their localization was compared with the previously described PVM protein exported protein-1 (EXP-1) (Simmons *et al.*, 1987). It is thus possible that S7 and S3 are secreted during sporozoite invasion and may become associated with the PV of the liver stages, but this remains to be determined.

Differential expression of a single genome that manifests itself in the distinct unicellular *Plasmodium* life cycle stages, each uniquely adapted to its respective host tissue, is a poorly understood phenomenon. We established that sporozoites differ significantly in gene expression from merozoites and that there are significant differences with regard to the composition of their apical organelles. This will certainly facilitate a better understanding of the unique aspects of sporozoite-target cell interactions and sporozoite transformation into liver stages.

Experimental procedures

Parasites

Anopheles stephensi mosquitoes were reared at 28°C and 75% humidity under a 14 h light/10 h dark cycle, and adults were fed on a 10% sucrose solution. Four- to 5-day-old female mosquitoes were blood fed on anaesthetized Balb/cByJ mice that had been infected with the *P. yoelii* strain 17XNL. Rodents were assayed for parasitaemia by blood smear and the abundance of gametocyte-stage parasites capable of exflagellation by wet mount. After the infective blood meal, mosquitoes were maintained at 24°C, 80% humidity.

Generation of subtraction libraries

On day 14 after feeding, 1.8×10^6 *P. yoelii* sporozoites from

salivary glands of infected *A. stephensi* mosquitoes were obtained. The sporozoites were purified over a DEAE-cellulose column resulting in 5.2×10^5 highly purified parasites. Poly(A)⁺ RNA was isolated from these sporozoites using oligo(dT) columns (Invitrogen). The poly(A)⁺ RNA was used as a template for first-strand cDNA synthesis and one subsequent round of amplification using the SMART-PCR cDNA synthesis kit (Clontech). This cDNA population was used as the tester population. A control reaction without reverse transcriptase did not result in detectable amplification, confirming the absence of genomic DNA contamination. For the driver population, we combined poly(A)⁺ RNA isolated from around 5×10^6 *P. yoelii* gradient-enriched schizont-stage parasites ($\geq 98\%$ schizonts), obtained after removal of leucocytes and thrombocytes by Plasmodipur filtration (Eurodiagnostics) and subsequent saponin lysis. This preparation was mixed with 50 dissected uninfected salivary glands. Next, the first-strand cDNA synthesis and one round of cDNA amplification (28 cycles) was performed. Suppression subtractive hybridization was achieved using the PCR-Select cDNA subtraction kit (Clontech). The subtracted cDNA population was ligated into vector pCR2.1-TOPO (Invitrogen) and transformed into *Escherichia coli* TOP10 competent cells (Invitrogen). Sequencing was done at a DNA sequencing facility (Rockefeller University) by BigDye terminator cycle sequencing with the M13 reverse primer.

Gene identification and sequence analysis

The subtracted *P. yoelii* cDNAs were clustered using the program SEQUENCHER, version 3.1.1. The consensus sequences of clusters containing two or more cDNA sequences were used to retrieve the complete genes from the *P. yoelii* genome database at the Institute for Genomic Research (<http://www.tigr.org>) by BLASTN search. The *P. yoelii* proteins were predicted using NCBI ORF FINDER (<http://www.ncbi.nlm.nih.gov/gorf>). Protein sequences were then used to search the GenBankTM non-redundant protein database and the PlasmoDB automated predictions of *P. falciparum* proteins (<http://www.PlasmoDB.org>) using BLASTP. Functional predictions were obtained using the translated protein sequence to screen the Pfam database (<http://www.pfam.wustl.edu/>). The results were confirmed by modular architecture research tool analysis (SMART) domain profile search (smart.embl-heidelberg.de). S genes for which our predicted protein sequence was different from the annotated sequences available through GenBankTM were submitted to GenBankTM and are available with the accession numbers: S4, AY427752; S6, BK001659; S10, BK001660; S13, BK001661; S15, BK001662; S21, BK001663; S22, BK001664; S23, BK001665; S24, BK001666.

Dot-blot analysis

A dot-blot analysis was used to confirm the expression of tagged genes in each population. Representative cDNA inserts were amplified by PCR. Approximately 1 µg of the PCR product was spotted on a nylon membrane, denatured and hybridized to stage-specific probes. Probes were prepared from cDNA of salivary gland sporozoites and mixed blood stages (1% gametocytes) and labelled using digoxige-

nin-dUTP (Roche Molecular Biochemicals). In the reverse analysis, cDNAs from salivary gland sporozoites and mixed blood stages were separated on agarose gels, blotted and hybridized to gene-specific probes.

Reverse transcriptase-PCR

Total RNA was isolated from salivary gland sporozoites and mixed blood stages using Trizol (Invitrogen). RNA was treated with DNase I (Invitrogen) to remove contaminating genomic DNA and used as template in RT-PCR with gene-specific oligonucleotide primers. Sequences are as follows: S1 (sense, 5'-TCTTTGTGGATATGCCCATGG-3'; antisense, 5'-TCTGCATTCCACTGATTCATACA-3'), S2/CSP (sense, 5'-AGCCCAAAGAACTTAAACGAGC-3'; antisense, 5'-GCCAAGTAATCTGTTGACTATATTCGA-3'), S3 (sense, 5'-CTTGCTTGATGCACCCTGAAG-3'; antisense, 5'-GGTATGGA TTTTCGACTGGG-3'), S4 (sense, 5'-TTTCTTGAAGGTGG AGTCAATC-3'; antisense, 5'-TTTCATTGGTAATTGTTTCG GCT-3'), S5 (sense, 5'-CATCATGGGACGAATTACCACA-3'; antisense, 5'-CCTGGAAGGCTACTGCGG-3'), S6 (sense, 5'-GGGTACATGTGCTGGCTATAA-3'; antisense, 5'-ATC TCTACTTTTTCTTTGTTCTTCCGTG-3'), S7 (sense, 5'-ATG TGAACTAGCTGCTAAACACCA-3'; antisense, 5'-ACATCC TTATTTTCATCTAAAGTCTGGAC-3'), S8/TRAP (sense, 5'-CATCTGACTCAGAAGTAGAATATCCAAGA-3'; antisense, 5'-TATGGGTATTACCTGGTGATGG-3'), S12 (sense, 5'-TGT GTATAACAATGAAAAGAGCCAGTA-3'; antisense, 5'-TGGT TTCTAAATGGTTATCTGTTTCATTT-3'), S13/PPLP1 (sense, 5'-AATGATGCAGATAACGAAGGTGG-3'; antisense, 5'-TAT GTTTATCAATTACATTTCTGGGTTTT-3'), S19 (sense, 5'-CGAGATTATGATCCTGAAAAAGGATATA-3'; antisense, 5'-TGACATCTCCAAGATCCATAACCTC-3'), S21/TRSP (sense, 5'-GATGGACAGAATGGTCGCAGT-3'; antisense, 5'-TTTA GAACAGAGTAATGAATAATAGGGTTACTAC-3').

Quantitative real-time RT-PCR

Two million salivary gland and 2 million oocyst sporozoites of *P. yoelii* were purified over a DEAE-cellulose column to remove contaminating mosquito tissue, yielding 500 000 highly purified parasites for each population. Total RNA was isolated using the RNeasy Mini kit (Qiagen). RNA was treated with DNase I (Invitrogen) to remove contaminating genomic DNA. Twenty nanograms of RNA for each sporozoite population was used as a template in first-strand cDNA synthesis, using the TaqMan® reverse transcriptase kit (PE Applied Biosystems). Gene-specific oligonucleotide primers were designed using the PRIMER EXPRESS software (PE Applied Biosystems) and are the same as those used for regular RT-PCR. PCR fragments were cloned into plasmid pCR4 (Invitrogen). Each plasmid construct was used in a 10-fold dilution series (10 copies to 10^6 copies, each in triplicate) to determine a standard curve. The standard curve plots the threshold value (Ct), defined as the cycle number at which reporter dye fluorescent intensity increases over background, over plasmid copy number. Absolute transcript copy number for each gene is calculated based on the external standard curve. Real-time RT-PCR amplification was done in a GeneAmp® 5700 sequence detection system (PE Applied Biosystems) using the double-stranded DNA-binding probe SYBR

Green I® (PE Applied Biosystems). Reactions were subjected to one cycle of 10 min at 95°C and 45 cycles of 15 s at 95°C, 1 min at 60°C. Real-time RT-PCR experiments were done in triplicate.

Recombinant protein expression and purification

A GST fusion protein corresponding to amino acids 78–224 of PyS3 was generated with two oligonucleotides containing a 5' *Eco*RI site and a 3' *Xho*I site (sense, 5'-gaattcGAAGTC CGAGAAAAATTTGGAATTAG-3'; antisense, 5'-ctcgagTTATA TGTATGGGTCAAATGGTTATC-3'). A GST fusion protein corresponding to amino acids 83–216 of PyS7 was generated with two oligonucleotides containing a 5' *Eco*RI site and a 3' *Xho*I site (sense, 5'-gaattcTCTCATAATAAAGGAAGA CATGATTGG-3'; antisense, 5'-ctcgagTTATTGTTCTTTAAGA AAATGCTCCACGCC-3'). A GST fusion protein corresponding to amino acids 23–75 of the N-terminal portion of PyS21/TRSP (see Fig. 4) was generated with two oligonucleotide primers containing a 5' *Eco*RI site and a 3' *Xho*I site (sense, 5'-gaattcGAATTCGCCCAATACAATGATACATTCTTA-3'; antisense, 5'-ctcgagCTCGAGTTAACTTAGCATTTCTTTTTCT TTTTT-3'). After restriction endonuclease digestion, the PCR products were inserted in frame into the pGEX-4T-1 expression vector (Pharmacia). *E. coli* BL21-rosetta cells (Novagen) were transformed with the recombinant expression vector. Fusion proteins were purified on glutathione Sepharose 4B (Pharmacia) and eluted with reduced glutathione. Fifty micrograms of the S7–GST fusion protein, suspended in phosphate-buffered saline, pH 7.4 (PBS), with Freund's adjuvants was injected subcutaneously into female BALB/cByJ mice at 3 week intervals. Two weeks after the second boost, blood was collected and serum was isolated. Polyclonal immune serum to the S3–GST fusion protein and the S21–TRSP–GST fusion protein was prepared by immunization of rabbits using the same immunization schedule as for S7 but 100 µg of each fusion protein. Normal mouse serum was taken from the non-immunized BALB/c mice, and normal rabbit serum was taken from the non-immunized rabbits before immunizations.

Immunofluorescence microscopy

For immunofluorescence, salivary gland sporozoites were isolated and air dried on slides. The parasites were fixed with 2% paraformaldehyde (Sigma) and incubated with antisera. PyS7 was detected with the mouse polyclonal anti-S7–GST fusion protein antibody (1:500). PyS3 was detected with the rabbit polyclonal anti-S3–GST fusion protein antibody (1:500). PyS21/TRSP was detected with the rabbit polyclonal anti-S21–TRSP–GST fusion protein antibody (1:500). Bound antibodies were detected using Alexa Fluor 488- and Alexa Fluor 594 (Molecular Probes)-conjugated anti-mouse and anti-rabbit secondary antibodies. Bound anti-TRAP and anti-CSP was detected using Alexa Fluor 594 (Molecular Probes)-conjugated anti-mouse secondary antibodies.

Immunoblotting

Sporozoite and mixed blood-stage proteins were solubilized

in sample buffer and separated on 10% polyacrylamide gels. Molecular weights were verified using a prestained molecular mass marker (Bio-Rad). Proteins were transferred to nitrocellulose (Bio-Rad) membranes by electroblotting. After transfer, membranes were blocked in 1× TBS–5% milk and then incubated with a 1:1000 dilution of anti-S21–TRSP antisera or a 1:3500 dilution of anti-S3 antisera. Membranes were washed and then incubated for 1 h at room temperature with a horse-radish peroxidase-conjugated secondary antibody. Membranes were washed again, and immunostained proteins were visualized with enhanced chemiluminescence detection (Pierce).

Acknowledgements

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We have created a second *Plasmodium berghei* parasite that is genetically attenuated at the sporozoite stage. This was done by targeted disruption of a gene (knockout, ko) named *S7/UIS3* (the mutant *P. berghei* parasite clone is herein referred to as: *S7/UIS3 ko*). The gene disruption (Ménard and Janse, 1997) was done using insertion of a selectable marker into the *S7/UIS3* locus.

The *S7/UIS3* (accession number: EAA22537) gene was previously identified by using differential gene expression screens with the related rodent malaria parasite species *P. yoelii* (see appended paper in press).

The *S7/UIS3* gene is not expressed in the parasites blood stages (see appended paper in press). *S7/UIS3ko* parasites are not defective in blood stage replication. The *S7/UIS3ko* parasites also show normal development of the mosquito stages of the parasite and develop normal numbers of sporozoites in the mosquito salivary glands. *S3/UIS4ko* sporozoites also infect the liver of experimental mice. However, *S3/UIS4ko* parasites are defective in complete liver stage development and thus unable to initiate subsequent blood stage infection. We will test *S7/UIS3* for their capacity to induce sterile protection against subsequent infectious wildtype sporozoite challenge. We have identified a *P. falciparum* *S7/UIS3* ortholog (Pf13_0012).